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## ABSTRACT

The infrared pit organs of pit vipers and pythons were studied with emphasis on blood flow in the capillary bed and its possible role as a cooling mechanism for the pit receptors stimulated by infrared (= heat) radiation to improve image resolution. Fluorescein isothiocyanate (FITC) dextran together with fluorescent microspheres was introduced into the blood stream and pit blood flow was viewed with a fluorescence microscope and CCD camera. Output of the camera was recorded before, during, and after infrared laser stimulation with a high-speed video system at 250 frames/sec. The FITC rendered all the pit blood vessels visible without interfering with the visibility of the microspheres. After recording, the course and speed of individual microspheres were traced with proprietary software and analyzed by one-way ANOVA and other statistical tests. In pit areas directly irradiated by the laser, blood flow speed was remarkably increased in the capillaries, but not in the arterioles or venules, during stimulation at a statistical significance of  $P=0.0002$  as compared with the speed before and after stimulation. This phenomenon lends credibility to the theory that capillary blood flow in the pits is used directly by the receptors as a coolant mechanism for fine-tuning image resolution.

## GENERAL INTRODUCTION

Boid snakes (i. e. boas and pythons) [12, 13] and crotaline snakes (i. e., pit vipers such as rattlesnakes and copperheads) are the only vertebrates known to have image-forming sensory organs that utilize the long wavelengths of the infrared spectrum, in contrast to the shorter wavelengths perceived by the eyes. These organs, called pit organs, are most highly evolved in the venomous crotaline snakes or pit vipers [10]. In these animals the pit organs are a pair of deep pits in the face between the nostril and the eye. Inside the pit a thin (10-15 micrometers) membrane is suspended, dividing the organ into a closed inner chamber and an outer chamber open to the outside with the opening facing more or less forward. This pit membrane functions as an infrared-sensitive retina. It is concave, and suspended in such a way that part of it is facing forward and part of it facing sideways. The forward-facing part may play the same role as the fovea in the eye [2].

The pit membrane is innervated by 3 branches of the trigeminal nerve, each of which feeds a discrete, clearly delineated area of the membrane [9]. Nerve impulses generated in the membrane travel through 2 trigeminal ganglia, the ophthalmic and the maxillomandibular ganglia, to a dedicated tract (the lateral descending trigeminal tract, Lttd) and nucleus (LTTD) in the medulla oblongata, thence to a second relay nucleus, the reticularis caloris, and finally to the optic tectum, where infrared neurons make contact with visual neurons and the 2 modalities of information are integrated [6, 8, 10].

In the pit membrane itself, the receptors consist of discrete masses formed by free nerve endings and Schwann cells, and are called terminal nerve masses, or TNMs. The TNMs are arranged in a 2-dimensional array just beneath the surface of the pit membrane, and are packed with mitochondria to an extent not seen in any other sensory organ [3]. The precise molecular mechanism of impulse generation is not yet clear, but it is known for certain that impulses are generated by any change in the temperature of a TNM [10].

The mouth of the pit is smaller than the pit membrane, so that a pattern of light and shadow moves across the array of TNMs whenever an infrared object moves through the field of view of the pit. This causes differential firing in individual TNMs as the pattern moves and changes. Thus, the need arises for a cooling system to continually return excited TNMs to a basal temperature; otherwise any image produced on the array would be extremely blurred because of the afterimage effect.

It is hypothesized that the dense capillary network provides for this need [2]. The network has been visualized by injection of India ink and by resin corrosion casts, in combination with succinate dehydrogenase staining of the TNMs. The vasculature is also 2-dimensional like the TNM layer. Each capillary loop encloses 2-5 TNMs in such a way that all TNMs are in contact with the vasculature. The forward-looking areas of the "fovea" have a denser network, i. e., fewer TNMs per loop, than side-looking areas, causing the fovea to be more sensitive and have better image resolution than the rest of the membrane.

A laser Doppler blood flow meter has been used to measure blood flow changes in response to various infrared stimuli [5]. Local blood flow (i. e., in the immediate vicinity of the stimulus) responds with characteristic patterns and

with an extremely short response time of about 1 msec. The blood flow patterns followed the tonic-phasic firing patterns of infrared neurons recorded in electrophysiological experiments. This, and the short latency of the responses have led to the hypothesis that the receptors themselves signal the local vasculature to increase or decrease blood flow in response to their need for heat exchange.

The morphology of resin casts of the capillary vasculature in the snake retina, scales, and pit membrane has been compared with a scanning electron microscope. Capillaries in the retina and scales are smooth tubes, but those of the pit membrane are twisted and convoluted. It is hypothesized that this is an adaptation allowing for expansion of the capillary to increase blood flow readily [2].

Capillaries do not have smooth muscle in their endothelium, but immunohistochemical techniques have demonstrated smooth muscle alpha-actin and desmin in pit membrane capillary pericytes. These pericytes, in contrast to those of other capillaries, can be seen constricting the capillaries with processes extended at right angles to the long axis of the vessels [11]. Therefore it is reasoned that the TNMs release a neuromodulator such as nitric oxide which could cause relaxation of local pericytes to increase capillary diameter and speed up blood flow as needed. Increases in blood flow in response to a physiological stimulus such as moving a hand in front of the pit can be easily recognized on a television monitor attached to a CCD camera mounted on a dissecting microscope.

## OBJECTIVES OF THE PRESENT PROJECT

1. Investigate the role of the unique capillary network of the pit membrane (pit fundus in boids) in controlling infrared reception. This was given highest priority among the objectives, because this aspect has the most salient possibility for practical application to the field of miniturized, ambient-temperature infrared imaging.

2. Determine the precise modality of the nerves innervating the infrared pit

organs of crotaline and boid snakes: whether myelinated sensory A-delta fibers, unmyelinated sensory C fibers, sympathetic nerve fibers, or parasympathetic nerve fibers.

3. Determine the distribution of each nerve modality in the pit membrane (boids: pit fundus) that serves as an infrared retina.

4. Investigate in particular the role of C fibers, sympathetic fibers, and parasympathetic fibers in relation to infrared reception by the A-delta fibers.

5. Investigate how each nervous modality influences blood flow in the capillary network.

6. Investigate the nervous pathways through which the infrared reception controls behavior of the snake, particularly the recognition and acquisition of prey.

## MATERIALS AND METHODS

Animals used were the pit vipers *Gloydus* (= *Agkistrodon*) *blomhoffii* and *G. brevicaudus* and the ball python *Python regius*.

As a representative boid snake, the morphology of the capillary bed of the pit fundus of the ball python was studied for comparison with data previously obtained for the pit vipers. India ink was injected and the results studied with light microscopy. Immunohistochemical staining was performed and studied with a confocal laser microscopy. Resin corrosion casts were made of the blood vessels and studied with a scanning electron microscope. Finally, ultrathin sections were cut perpendicularly to the pit fundus and studied with a transmission electron microscope.

Doppler blood flow measurements were also made in living pythons and the data compared with data previously obtained from pit vipers.

In the pit vipers, double staining immunohistochemistry and transmission electron microscopic montages were used to examine the composition and nature of nerve bundles serving the pit organs. Nerve bundles containing unmyelinated fibers were treated with capsaicin in an attempt to determine the modality of such fibers.

Previous studies showed the presence of a communicating branch from the facial nerve to the deep branch of the trigeminal maxillary nerve, which is one of the most important sensory components of the pit infrared system. Therefore tracer techniques and immunohistochemistry were used to map the afferent and efferent nerve projections of the facial nerve in the pit vipers.

A combination was developed of high-speed video recorders (up to 1000 frames/sec), image-intensified CCD cameras attached to a dissecting microscope with coaxial illumination for fluorescence, and laser markers and stimulators for quantifying patterns of blood flow. A hardware interface was also constructed to allow integration of a stimulus marker into the high-speed microscopic image of blood flow in the pit membrane or fundus. Experiments were done to find the best way to introduce fluorescent microspheres and fluorescent dextrans into the bloodstream with a minimum of trauma to the animal. The prime candidate was injection of saline containing 3-micrometer microspheres and FITC-labeled dextran into the proximal tip of the liver.

Stimulation of the pit membrane was done with an 810 nm infrared diode laser beamed into the pit through an optic fiber probe. The laser spot was first aimed with a visible laser (630 nm) projected through the same probe. After aiming, the probe was switched to the output of the infrared laser. The video recorders permitted a maximum recording time of 8 sec, so the interface was set to record a time course of 2 sec stimulus off, 4 sec stimulus on, and 2 sec off.

Recordings were downloaded into a computer, and the courses of individual microspheres through arterioles, capillaries, and venules were traced with proprietary software in the intervals before, during, and after stimulation. Tracings were done separately in the area illuminated by the infrared beam, in areas adjacent to the stimulated area, and in areas relatively far removed from the stimulated area.

Proprietary software was used to trace the paths of individual microspheres from the arterioles, through the capillary bed, and out through the venules. Pathways, speeds, distances traveled, and other statistical parameters were recorded, tested by one-way ANOVA and other statistical procedures, and graphed. For visualizing the capillary bed, experiments were also done with backlighting the pit membrane via an optic fiber inside the mouth of the snake.

## RESULTS/NEW FINDINGS

1. In contrast to the single pair of facial pits in pit vipers, ball pythons have pits in the labial scales: 5 in the upper labials on both sides and 2 in the lower labials, for a total of 14.
2. The boid capillary bed consisted of a forest of interconnected loops oriented vertically to the TNM layer at the surface of the pit fundus. The vertex of each loop was expanded to a dome nearly twice the diameter of the vertical leg of the loop, and positioned directly beneath a TNM or TNMs. Furrows could be seen on the surface of the domes, presumably caused by constricting processes of blood flow-controlling pericytes, as seen in pit vipers. Part of one such pericyte was actually observed.
3. Doppler blood flow measurements ball pythons gave results virtually identical to those observed in pit vipers. Stimulus "on" produced a sharp peak response, with a latency of 1 msec. The response dropped to a plateau level during continued stimulation, and produced another sharp peak at stimulus "off".



4. In the 3 main nerve bundles serving the pit organs, myelinated A-delta fibers totaled 2,200-3,700 and unmyelinated fibers totaled 2,400. Two accessory bundles were also discovered composed almost entirely of unmyelinated fibers with a smattering of myelinated fibers. The total of unmyelinated fibers in the accessory bundles averaged 3,300. In the 5 nerves bundles, some of the unmyelinated fibers contained the neuromodulator substance P (SP), some vasoactive intestinal polypeptide (VIP), and the rest contained both SP and VIP. All three types were observed within the pit membrane and around its periphery. After treatment with capsaicin, the fibers inside the pit membrane disappeared, but those at the periphery remained.

5. The facial motor neurons controlling the jaw opening muscles were discovered to have many dendrites projecting toward the nucleus reticularis caloris, which is a relay nucleus for ascending infrared information.

6. Injection of FITC dextran allowed good fluorescent visualization of the vasculature inside the pit membrane. It became possible to distinguish clearly arterioles, venules, and capillaries, without obscuring the brighter fluorescence of simultaneously injected fluorescent microspheres. The most practical route of injection proved to be the vena cava rather than the liver itself.

7. There was difference among the parameters recorded for individual microspheres, but the general pattern was a speedy entrance via an arteriole, a slow winding passage through the capillaries, and acceleration again as the microsphere exited via a venule.

8. In the pit capillaries, there was a remarkable increase in blood flow speed during infrared stimulation, in comparison between periods before and after stimulation. This increase was extremely significant in the area directly stimulated ( $P = 0.0002$ ), but became less and less evident as the point of observation was moved farther and farther away from the stimulated area.

9. Increase in blood flow speed was notable only in the capillaries. Such increase was not observable in the arterioles or venules.

## DISCUSSION

The results of the python experiments showed that, despite obvious morphological differences from pit vipers, the python pits function in a similar manner as infrared imaging eyes, and contain the essential elements of the pit viper infrared eyes: i. e., mitochondria-packed nerve terminals close to the body surface (the TNMs) that produce action potentials when heated by impinging radiation, and a rapidly acting cooling system (the specialized capillary bed) that is under direct control of the TNMs themselves (via the pericytes) [4, 5].

The results of capsaicin treatment of unmyelinated fibers, whereby such fibers within the pit membrane were eliminated whereas those at the periphery remained, suggest that the fibers within the pit membrane are sensory fibers, and that those at the periphery are autonomic fibers. The precise role of these fibers in infrared imaging and blood flow control needs further investigation.

The observation of facial nerve motor neurons projecting dendrites toward the nucleus reticularis caloris raises the possibility of a reflex loop for control of jaw opening by infrared information. Further study should be done to search for synaptic contacts between these dendrites and processes of reticularis caloris neurons.

The statistically significant increase in blood flow speed in the pit capillaries during infrared stimulation, versus the lack of such notable increase in the arterioles and venules, bolsters the previously advanced theory of local control of capillary blood flow by the TNMs, and validates the more averaged results of Doppler blood flow measurements. In light of previous observations of the tight conjunction between individual TNMs and capillaries, the blood flow increase during stimulation also bolsters the theory that this occurs for the purpose of receptor cooling to avoid afterimage, in addition to the general purpose of alimentation and oxygen supply.

It was not possible to predict the path that a microsphere would take when it entered the capillary bed at a given anastomosis in an arteriole, even during "resting" periods between stimulations. Presumably the blood flow carrying a given microsphere is shunted here and there by the capillary pericytes and sphincters in response to excitation of the infrared receptors by background radiation. Analysis of the shunting and velocity changes induced by controlled stimulation may provide hints for practical application in the field of infrared imaging at ambient temperatures.

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PAPERS RESULTING FROM THIS PROJECT:

Hisajima T, Kishida R, Atobe Y, Nakano M, Goris RC, Funakoshi K: Distribution of myelinated and unmyelinated nerve fibers and their possible role in blood flow control in crotaline snake infrared receptor organs. J. Comp. Neur. 449:319-329 (2002).

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